सत्यमेव जयते

INTELLECTUAL PROPERTY **INDIA**

GOVERNMENT OF INDIA
MINISTRY OF COMMERCE & INDUSTRY,
PATENT OFFICE, DELHI BRANCH,
W - 5, WEST PATEL NAGAR,
NEW DELHI - 110 008.

CERTIFIED COPY OF PRIORITY DOCUMENT

I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application*** *and* ***Complete Specification*** *filed in connection with Application for Patent No.* ***534/Del/2003*** *dated* ***31st March 2003.***

Witness my hand this 12th day of July 2004.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

0534-DEL-03

FORM 1
THE PATENTS ACT, 1970
(39 OF 1970)
APPLICATION FOR GRANT OF PATENT
(See Sections 5(2), 7, 54 and 135 and rule 33A)

31 MAR 2003

1. We COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH, Rafi Marg, New Delhi- 110001, India, an Indian registered body incorporated under the Registration of Societies Act (Act XXI of 1860);
2. hereby declare:

(a) that we are in possession of an invention titled: An improved economical process for the isolation of hepatoprotective agent 'Oleanolic Acid" from Lantana Camara

(b) that the Provisional / Complete specification relating to this invention is filed with this application;
(c) that there is no lawful ground of objection to the grant of patent to us;

3. further declare that the inventor(s) for the said invention is / are: **Santosh Kumar Srivastava, Merajuddin Khan** and **Suman Preet Singh Khanuja** all of Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow, India, 226015 all Indian citizens.

4. We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows: NOT APPLICABLE

5. We state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which we are the applicant:
   (a) Patent application no.:
   (b) Patent application date:
6. We state that the application is divided out of our application, the particulars of which are given below and pray that this application deemed to have been filed on ----------under section 16 of the Act.
   (a) Patent application no.:
   (b) Date of filing provisional and /or complete specification ----------an d ----------
7. That we are the assignee of the true and first inventor(s).

8. That our address for service in India is as follows:

Head, IPM Division, CSIR,
INSDOC Building, 14 Satsang Vihar Marg,
New Delhi – 110067.
Phone: 696 2560, 696 8819; Fax: 696 8819.

PTO

9. Following declaration was given by the inventor(s) :
I / We the true and first inventor (s) for this invention declare that the applicants herein is / are my/our assignee:

Dated this 31st day of March 2003

Name (in full with expanded initials)	Signature of true and first inventor(s)
Santosh Kumar Srivastava	Santosh Kumar Srivastava
Merajuddin Khan	Merajuddin Khan
Suman Preet Singh Khanuja	Suman Preet Singh Khanuja

10. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

11. Followings are the attachments with the application:
(a) ~~Provisional~~ / Complete specification (3copies).
(b) Drawings (3 copies).
(c) Priority document (s).
(d) Statement and Undertaking on FORM-3.
(e) Power of authority.
(f) Fee Rs. 5000/- in Cheque no.: 868342 dated: 25.3.03 on State Band of India, New Delhi Main Branch, Parliament Street, New Delhi-110001.

We request that a patent may be granted to us for the said invention.

Dated this 31st day of March 2003

(
SCIENTIST
Intellectual Property management Division,
Council of Scientific and Industrial Research.

To,
The Controller of Patents,
The Patent Office, New Delhi.

FORM 2

THE PATENTS ACT -1970

(39 of 1970)

COMPLETE SPECIFICATION

(See Section 10)

AN IMPROVED ECONOMICAL PROCESS FOR THE ISOLATION OF HEPATOPROTECTIVE AGENT "OLEANOLIC ACID" FROM *LANTANA CAMARA*

COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

Rafi Marg, New Delhi -110 001, India, An Indian Registered Body incorporated under the Registration of Societies Act (XXI of 1860)

The following specification particularly describes the nature of the invention and the manner in which it is to be performed

The present invention relates to an improved and economical process for the isolation of hepatoprotective agent "oleanolic acid" from *Lantana camara*. oleanolic acid [(3-O-β-hydroxy-olea-12-en-28-oic acid (**1**)] is a triterpenoid compound. which exist widely in natural plants in the form of free acid or aglycones for triterpenoid saponins. Oleanolic acid has been isolated from more than 120 plant species. It has been identified as the main bioactive constituent of the medicinal plants used in folk medicine such as *Aralia chinensis*. var. nuda nakai, *Beta vulgaris* L. var. cicla L., *Swertia mileensis. Swertia japonica. Tetraponax papyriferum, Panax ginseng* used in hepatoprotection: *Ligustrum lucidum. Luffa cyclindrica. Oleandra neriifolia, Sapindus mulcorossi* used in anti-inflammation and *Gonoderma lucidum* and *Glechoma hederacea* used for anticarcinogenic activity and antitumor promotion.

Oleanolic acid. as such has been reported to exhibit potent hepotoprotective activity. It decreases CCl_4-induced liver parenchymal cell necrosis, steatosis and degeneration plus alcohol-induced chronic cirrhosis. It is as such marketed in China for human hepatitis. Similarly oleanolic acid has also shown significant anti-inflammatory activity by inhibiting raw paw edema produced by dextran and by suppressing adjuvant induced arthritis in rats and mice. It is also important to note that oleanolic acid also inhibits tumor initiation and tumor promotion. Treatment of rats with oleanolic acid (200 ppm) in diet for 3 weeks decreases the incidence and multiplicity of azoxymethane-induced intestinal tumor. Oleanolic acid also showed significant hypolipidemic and anti-atherosclerotic properties. Treatment of experimental hyperlipidemic rats with oleanolic acid (50mg/kg, P.O. for 9 days) decreases the elevated blood cholesterol and β-lipoprotein levels by more than 40%. Cosmetic and pharmaceutical preparations of **1** have been patented in Japan for use in skin care and non-lymphatic leukemia.

Apart from the above. oleanolic acid has also shown antiulcer, antimicrobial, hypoglycaemic activity, protection against cyclophosphamide induced toxicity, anticarcinogenic and antifertility activities etc. It was observed that although oleanolic acid has been isolated from more than 120 plant species however. due to the poor yield and tedious column chromatographic separation procedures of the bioactive constituent from *Panax ginseng, Araiia chinensis, Eugenia Jaumbolana, Calendula officinalis. Gonoderma lucidum. Oleandra neriifolia* (Plants used in folk medicines) and most of the other plant species. this bioactive constituent has become

an expensive pharmaceutical compound. This prompted us to search for an inexpensive, easily available, wildly growing and rich source of oleanolic acid and develop an easy and economical process for the isolation of this important therapeutic agent so that it can be brought under the reach of common masses.

On going through the literature, it was observed that sugar beets may be an inexpensive, easily available source of oleanolic acid. An extraction procedure for oleanolic acid has also been patented from Sugar beet (**"Extraction of oleanolic acid from Sugar beets for treatment of liver failure"**, Yabuchi *et al.* 1988, chemical Abstract **108**, 82082p; Yabuchi *et al.* 1987, Japanease pat. No. 62126149). This process involves crude oleanolic acid preparation from 1Kg each of sugar beet roots and leaves. For further purification, the crude preparations were extracted with MeOH, treated with HCl, and subjected to column chromatographic separation. The recovery rate from crude preparation was only 66.3%.

The method described above suffers from a number of disadvantages. The biggest disadvantage of the above method is the very low (exact yield not available) concentration of oleanolic acid saponin in both leaf and roots of sugar beets. The second disadvantage of the above process is that oleanolic acid has not been isolated as such, but was obtained after an extra and tedious acid hydrolysis step, which reduces the recovery of bioactive constituent by almost 33%. The third major disadvantage of the above process is that it utilizes column chromatographic separation for the isolation of oleanolic acid using various mixture of eluting solvents. Thus resulting in a tedious, time taking and expensive process for the isolation of oleanolic acid.

The other inexpensive, easily available and rich source of oleanolic acid is roots of *Lantana camara*. *L. camara* is a prickly climbing aromatic shrub of the family Verbenaceae. It is native to tropical America and was introduced in India as an ornamental and hedge plant, but now it has been completely naturalized and growing very wildly throughout India. A dense wild population of this shrub can be easily seen along the railway lines, forests and in almost all the wild places. It has also been recorded that different parts of the plant are rich source of various bioactive principles. In Africa, infusion of the leaves are used against rheumatism, asthama, cough and colds. The whole plant and its infusions are considered to be anti-pyretic, diaphoretic and anti-malarial. Recently an isolation procedure for oleanolic acid has been patented from the rootlets and root bark of *L. camara* ("**High concentration of**

hepatoprotective oleanolic acid and its derivatives in *Lantana camara* roots", Misra *et al.* 1997, *Planta Med.* **63:** 582, Misra *et al.* 1996, *Indian Pat.* No. 184489). This process involves extraction of rootlets and root bark with a mixture of three solvents. The crude so obtained is chromatographed on silica gel column and the oleanolic acid rich fractions are further purified on another column, thus resulting in the isolation of 1.47% of oleanolic acid.

The method described above suffers from a number of disadvantages. The biggest disadvantage of the above method is that it uses rootlets and root barks of *L. camara*. The rootlets are very small and few in *L. camara*, hence can not be obtained in sufficient amount for commercial purpose. Similarly the roots of *L. camara* are also small in size and are covered with very thin bark, hence peeling off the bark on commercial scale is neither possible nor it will be economical. It has been observed that rootlets and root barks in *L. camara* constitute not more than 20% of the total roots. Hence for obtaining 3.75Kg of rootlets and root bark, 18.75Kg of roots would have been certainly used, which gave only 55g of oleanolic acid in the above process. But if 18.75Kg of *L. camara* roots are processed according to our method it will give ~187.5g of oleanolic acid. In this way it is very clear that the yield of oleanolic acid by our process is 3.4 times more than the above process. The second disadvantage of the above process is that it utilizes mixture of three solvents for the extraction of plant material, which neither can be used again nor can be recycled. The third major disadvantage of the above process is that it utilizes repeated column chromatography on silica gel for the isolation of oleanolic acid using mixtures of eluting solvents, thus resulting in a tedious, time taking and enormous expensive procedure, which can not be economically viable.

The main object of the present invention is to provide an improved economical process for the isolation of oleanolic acid directly from the roots of *L. camara*, which obiates the draw backs of the existing processes.

Another object of the present invention is to completely avoid use of highly tedious, time taking column chomatographic purification process for the isolation of oleanolic acid from the roots of *Lantana camara*.

Still another object of the present invention is to provide an economical process for the isolation of oleanolic acid from the roots of *Lantana camara*.

Another object of the present invention is that it completely omit the use of highly tedious. time taking and expensive repeated column chromatographic purification process used in prior art processes.

Another object of the present invention is that it directly uses roots while the existing processes use only rootlets and rootbark. resulting in a yield advantages of 3.4 times.

Another object of the present invention single solvent can be used for the extraction of oleanolic acid the plant material. which can be reused and/or recycled However, the prior art process uses mixture of three solvents, which can neither be reused nor can be recycled.

Still another object of the present invention is that this process uses simple precipitation and crystallization processes for the isolation of oleanolic acid which are easy. less time taking and highly inexpensive.

Accordingly the present invention provides an improved and economical process for the isolation of oleanolic acid from the roots of *Lantana camara.* which comprises of drying, grinding and defattening of *Lantana camara* roots with light petroleum followed by over night extractions at room temperature (30-40°C) three times with a single solvent selected from CH_2Cl_2. $CHCl_3$. EtOAc, ether, acetone. MeOH. EtOH etc., removal of solvent under vacuum at 35-45°C, precipitation of crude extract and repeated partial crystallization of precipitate with a single solvent selected from CH_2Cl_2. $CHCl_3$. EtOAc, ether, acetone, MeOH, EtOH, H_2O and others resulting in the isolation of oleanolic acid with 1% yield..

In an embodiment of the present invention a varied range of defattening solvents petroleum ether, hexane, benzene, toluene and dichloromethane can be used.

In another embodiment of the present invention a varied range of fractionating solvents CH_2Cl_2, $CHCl_3$, EtOAc, ether, actone, MeOH, and EtOH can be used.

Still in another embodiment of the present invention a varied range of precipitating and crystallizing solvents CH_2Cl_2, CH_3CHCl_2, $CHCl_3$, EtOAc, ether, actone. MeOH. EtOH and H_2O can be used.

In another embodiment of the present invention which completely omit the use of highly tedious. time taking and expensive repeated column chromatographic purification process used in prior art processes.

In another embodiment of the present invention which directly uses roots while the existing processes use only rootlets and rootbark, resulting in a yield advantages of 3.4 times.

In another embodiment of the present invention wherein single solvent can be used for the extraction of oleanolic acid the plant material, which can be reused and/or recycled However, the prior art process uses mixture of three solvents, which can neither be reused nor can be recycled.

In another embodiment of the present invention uses simple precipitation and crystallization processes for the isolation of oleanolic acid which are easy, less time taking and highly inexpensive.

The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of present invention.

Example - 1

The fresh *Lantana camara* roots were collected from the field. The roots were first washed and made free from soil and other organic matters. The clean roots were chopped into small pieces and shade dried. The dried roots were powdered in a grinder. The powdered *Lantana camara* roots (700g) were first hot defatted with petroleum ether (bp 40-60°C and then extracted with dichloromethane (CH_2Cl_2). The extraction was carried out for 8 hrs till the material was completely exhausted. Removal of the solvent under vacuum at 40°C gave a brownish viscous mass. This was dissolved in excess of water and left over night at room temperature. The precipitate so obtained was filtered and the precipitate was crystallized with ether four times, which resulted in the isolation of oleanolic acid in 0.7% yield.

Example - 2

The powdered roots (2Kg) were first cold defatted with hexane and then extracted with MeOH over night four times at room temperature. Removal of the solvent was carried out under vacuum at 40°C. The crude extract was dissolved in excess of EtOAC and left overnight at room temperature. The precipitate was filtered and crystallized with MeOH. Precipitation and crystallization processes were repeated 4 times, which resulted in the isolation of oleanolic acid in 0.85% yield.

Example - 3

The powdered roots (1.5Kg) were first hot defatted with petroleum ether (bp 40-60°C) and then extracted with acetone. The extraction areas carried out for about 8 hrs till the material was completely exhausted. Removal of the solvent under vacuum at 40°C gave a brownish viscous mass. This was dissolved in excess of dichloromethane by heating and then left over night at room temperature. The precipitate so obtained was filtered and crystallized with ether thrice, which resulted in the isolation of oleanolic acid in 0.65% yield.

Example -4

The powdered roots (3Kg) were first cold defatted thrice with petroleum ether (bp 40-60°C) at room temperature over night. The defatted material was then extracted with $CHCl_3$ four times at room temperature over night. Removal of the solvent was carried out under vacuum at 40°C. The crude extract so obtained was dissolved in acetone and left over night for precipitation. The precipitate so obtained was filtered and crystallized with EtOH. Precipitation and crystallization process were repeated 4 times which gave oleanolic acid in 0.8% yield.

Example - 5

The powdered roots of *L. camara* (5Kg) were defatted with hexane in cold thrice at room temperature. The defatted material was then extracted with EtOAc four times overnight at room temperature. The solvent was removed under vacuum at 40°C and the crude extract so obtained was dissolved in ether and left over night in refrigerator for precipitation. The precipitate so obtained was filtered and dissolved in MeOH for crystallization. Precipitation and crystallization process were repeated for 4 times, which gave oleanolic acid in 0.7% yield.

Example -6

The powdered roots of *L. camara* (10Kg) were defatted thrice in cold overnight with petroleum ether (bp 40-60°C) and then extracted exhaustively with EtOH four times overnight at room temperature. The solvent was removed under vacuum at 40°C and the crude was dissolved in $CHCl_3$ and left overnight for precipitation. The precipitate

so obtained was crystallized with MeOH. Precipitation and crystallization process were repeated 4 times. which gave oleanolic acid in 0.9% yield.

Advantages

1. The main advantage of our process is that it completely omit the use of highly tedious. time taking and expensive repeated column chromatographic purification process used in prior art processes.
2. The other major advantage of our process is that it directly uses roots while the existing processes use only rootlets and rootbark, resulting in a yield advantages of 3.4 times.
3. Single solvent can be used for the extraction of oleanolic acid the plant material, which can be reused and/or recycled However, the prior art process uses mixture of three solvents. which can neither be reused nor can be recycled.
4. The present process uses simple precipitation and crystallization processes for the isolation of oleanolic acid which are easy, less time taking and highly inexpensive.

Claims:

1. An improved and economical process for the isolation of oleanolic acid from the roots of *Lantana camara* which comprises of drying, grinding and defattening of *Lantana camara* roots with light petroleum followed by over night extractions at room temperature (30-40°C) three times with a single solvent selected from CH_2Cl_2, $CHCl_3$, EtOAc, ether, acetone, MeOH, EtOH, removing solvent under the vacuum at around 40°C, precipitation of crude extract and repeated partial crystallization of precipitate with a single solvent selected from CH_2Cl_2, $CHCl_3$, EtOAc, ether, acetone, MeOH, EtOH, and H_2O and others resulted in the isolation of oleanolic acid with 1% yield..
2. As claimed in claim 1 wherein defattening solvent is selected from, petroleum spirit, hexane, benzene, toluene and dichloromethane etc.
3. A highly improved process as claimed in claims 1 and 2 wherein fractionating, precipitating and crystallizing solvents are selected from dichloromethane, dichloroethane, chloroform, ethylacetate, diethyl ether, acetone, methanol, ethanol, H_2O and others.
4. A process as claimed in claims1 to 3 which completely omit the use of highly tedious, time taking and expensive repeated column chromatographic purification process used in prior art processes.
5. A process as claimed in claims 1 to 4 which directly uses roots while the existing processes use only rootlets and rootbark, resulting in a yield advantages of 3.4 times.
6. A process as claimed in claims 1 to 5 wherein single solvent can be used for the extraction of oleanolic acid the plant material, which can be reused and/or recycled However, the prior art process uses mixture of three solvents, which can neither be reused nor can be recycled.
7. A process as claimed in claims 1 to 6The present process uses simple precipitation and crystallization processes for the isolation of oleanolic acid which are easy, less time taking and highly inexpensive.

8. A highly improved isolation process of oleanolic acid from the roots of *Lantana camara* substantially as here in described with reference to the examples accompanying this specification.

Dated this 31st day of March 2003

Scientist
IPMD, CSIR

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